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STUDIES ON POLY-D-GLUTAMIC ACID PRODUCED BY *BACILLUS LICHENIFORMIS* NRRL B-571

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STUDIES ON POLY-D-GLUTAMIC ACID PRODUCED BY

BACILLUS LICHENIFORMIS NRRL B-571

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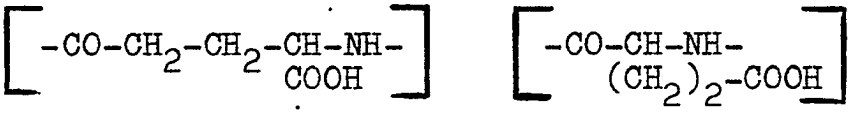
CHAPTER I

INTRODUCTION

Certain strains of various species of the bacterial genus *Bacillus* are able to produce a polypeptide which is composed solely of D (-) glutamic acid. Such a polypeptide, containing only a single kind of amino acid residue, is probably unique in nature. It presents some attractive features as a model for research in polyelectrolytes. Its production by bacteria provokes some intriguing questions. How is the polypeptide formed in nature, and why? Glutamic acid has two carboxyl groups per molecule. This fact leads to the question of which one of the two carboxyl groups is involved in the peptide bonds of a linear polypeptide such as natural poly-D-glutamic acid. The individual glutamic acid residues could be linked in either of the two following ways:



Gamma linkage Alpha linkage

The question of why the polypeptide is produced by bacteria has not yet been answered or even mentioned in the literature. One possible answer is presented in this dissertation along with some preliminary data supporting this suggestion.

The question of how the polyglutamic acid is formed has been answered satisfactorily for the time being by Thorne, et al. (1-5). However, the exact nature of the linkage in the polypeptide, involving as it may either the alpha or gamma carboxyl group, has not been unequivocally established. Dogmatic statements supporting both possible linkages have been printed (6,7). It is quite possible to pick the best of the evidence to support whichever view one prefers.

Ivanovics and co-workers (8,9,10) were the first to report the isolation of poly (-) glutamic acid from bacterial cultures and to prove its simple constitution, but they did not determine the nature of the linkage between the glutamic acid units. Bovarnick (6) and Bovarnick and Bovarnick (11) published results indicating the presence solely of gamma links in the polypeptide. These were results of attempted racemizations of the polypeptide in dilute alkali. No racemization occurred in any of the samples. Extrapolating from results of previous experiments in alkaline racemizations by Dakin (12) and by Bovarnick and Clarke (13), Bovarnick and Bovarnick concluded that only gamma

bonds were present. Dakin's work dealt with the racemization by alkali of hydantoins having a hydrogen atom on the alpha methylene carbon. The presence of a hydrogen in this position allows a keto-enol tautomerism to destroy the original optical activity. Bovarnick and Clarke showed that the alpha peptide link could undergo a double keto-enol tautomerism, the first step serving to reinforce the second step (racemization) by virtue of the formation of a system of conjugated double bonds.

$$\left[-NH-\overset{*}{C}HR-CO-NH-\overset{*}{C}HR-CO-\right]_X \leftrightarrows \left[-NH-\overset{*}{C}HR-COH=N-\overset{*}{C}HR-CO-\right]_X \leftrightarrows \left[-NH-\overset{*}{C}HR-COH=N-CR=COH-\right]_X$$

Racemization occurred immediately when hydantoins were mixed with the alkali. The peptides were also racemized, but much more slowly. Bovarnick presented experimental evidence for the complete racemization of aspartic acid (the next lower homolog of glutamic acid) in six proteins, gelatin, casein, bovine caseinogen, sheep caseinogen, hen albumin, and duck albumin, by exposure to dilute alkali for fifteen days, and for the complete racemization of glutamic acid in all of these proteins except gelatin (no racemization) and sheep caseinogen (partial racemization). He noted that the lack of racemization in these two cases may mean either that gamma glutamyl bonds are involved or that the glutamic acid moieties are terminal in the peptide chains. A complete lack of alkaline racemization in the case of a protein composed solely of glutamic acid units should indicate the

presence of gamma glutamyl bonds only.

In 1946, Hanby and Rydon (7) reported results of several different experimental approaches to the problem of linkage in polyglutamic acid isolated from B. anthracis and concluded that typical alpha peptide bonds were definitely present in poly-D-glutamic acid. In the Van Slyke analysis for amino-nitrogen, the standard reaction time is three minutes, but extensions of the reaction time do not usually change the results for a normal protein. Hanby and Rydon noticed that the apparent amino-nitrogen value on polyglutamic acid varied directly as the reaction time was increased. Apparently the effective molecular weight of the polypeptide was being decreased at a marked rate as the time of reaction with nitrous acid was increased. The samples of highest molecular weight seemed to be affected most, but for all samples the apparent molecular weight eventually approached a minimum, or limiting, value in the neighborhood of 5000. This was considered by Hanby and Rydon to mean that less easily hydrolyzed alpha peptide bonds were linking glutamic acid units in groups having a molecular weight of about 5000. These groups were then linked to each other by chains of glutamic acid units joined by gamma links. Furthermore, the poly-D-glutamic acid is rather rapidly hydrolyzed by acid. Since the gamma linkages are less stable to acid hydrolysis than are the alpha links and since compounds such as glutathione and glutamine, which involve gamma acyl-amino

bonds, are known to give high and varying Van Slyke amino-nitrogen values, the presence of gamma linkages is assumed in the explanation above. The long chain, or thread-like, structure of poly-D-glutamic acid is indicated by the fact that solutions of the polypeptide have high viscosities.

Since the alpha and gamma carboxyl groups have different acid strengths (pK_α 2.90, pK_γ 4.35), Hanby and Rydon were also able to show, by titrations, that the apparent ratio of free gamma carboxyl groups to free alpha carboxyl groups increased with decreased molecular weight of the samples titrated. The implication is that the higher the molecular weight, the greater is the number of gamma links with respect to the number of alpha links also present in the polypeptide.

TABLE 1

RELATIONSHIP OF MOLECULAR WEIGHT TO TYPE OF LINKAGE

Molecular Weight	Number Of Units	Ratio Of Free Carboxyl Groups
53,000	350	8:1 $\gamma:\alpha$
30,000	200	10:1 $\gamma:\alpha$
21,000	160	15:1 $\gamma:\alpha$
15,000	100	50:1 $\gamma:\alpha$

In 1952, Ciereszko and Crane reported in a brief note (14) an improved method of isolation of polyglutamic acid and the results of some sedimentation and titration

studies on it. They found an apparent pK of 3.9 which would indicate that the majority of the free carboxyl groups must be gamma groups. This would be expected from the values given in table 1. A pK of 3.9 would indicate a ratio of free gamma to free alpha carboxyl groups of just slightly greater than 2:1 or a molecular weight greater than 53,000. No molecular weight was given for the sample titrated, but with the improved method of isolation mentioned, less degradation and, consequently, higher molecular weight material would be expected. All previous methods of isolation had involved dialysis against acid, a process undoubtedly leading to considerable degradation. Sedimentation rates were given as 1.5 S and 1.05 S (Svedberg unit; 1×10^{-13} cm./sec./unit field) at pH 2.1 and 4.1, respectively.

Kovacs and co-workers reported in 1952 and 1953 (15, 16, 17) concerning a strictly chemical analysis of the type of bond present in natural poly-D-glutamic acid. They attempted the isolation and identification of the degradation products resulting from the Curtius and Hofmann reactions on the natural polypeptide. Methyl alcohol was used to esterify the free carboxyl groups of the polypeptide. For the Hofmann degradation, the polyester was easily converted to the polyamide with liquid ammonia. After oxidation with hypochlorite, the acid-aldehyde was isolated as the p-nitrophenylhydrazone of the aldehyde and shown to be

succinic half-aldehyde. The yield was quite small. This compound would result if the gamma carboxyl is tied up in the peptide links. The polyester was also treated to form the polyhydrazide. Reaction of the polyhydrazide with nitrous acid should give a polymer of α,γ-diaminobutyric acid. Hydrolysis should then yield the simple units of α,γ-diaminobutyric acid. Attempted isolation of this compound using flavianic acid as a specific precipitant was unsuccessful. The polypeptide used in this case was of about 9000 molecular weight, and apparently had only gamma bonds. These degradations were also attempted by Brown (18).

Waley (19) reported the synthesis of and studies on poly-(γ-L-glutamyl)-L-glutamic acid and the corresponding alpha linked isomer. These synthetic polymers had molecular weights of about 9000. The solubility of the synthetic gamma linked polymer in water was reported to be similar to that of the natural polypeptide, while the synthetic material containing alpha links was reported to be much less soluble. Infra-red studies showed absorption bands at 8.25, 8.88, 9.80 and at 7.12, 7.90, 8.58 and 12.70 for the gamma and alpha linked types of polypeptide respectively. The polypeptide from Bacillus licheniformis showed an absorption spectrum very similar to that of the synthetic gamma linked polymer. While the spectrum of the polypeptide from B. anthracis was poorly resolved, it "clearly" belonged to a

gamma linked type. The absorption band at 8.25μ is characteristic of the isolated acyl-amino grouping, which would be absent in alpha linked material. This absorption band was definitely present in the spectrum of the natural polypeptide however. Waley also reported a pK of 3.6 $\pm$ 0.1 for the synthetic gamma linked polymer. (Cf. Ciereszko and Crane (14) pK 3.9 for the natural polypeptide.) The molecular weights were determined by arylation with 2,4 dinitrofluorobenzene. One sample of polypeptide isolated from B. anthracis had a molecular weight of 180,000 or about 1260 glutamic acid residues! The average samples from B. licheniformis showed a molecular weight of about 90,000.

Bovarnick and Bovarnick (11) reported that in twelve hours 70-80% of the natural polyglutamic acid can be degraded to less than 3000 molecular weight by aqueous extracts or homogenates of all human tissues except muscle and plasma. Synthetic alpha linked polypeptide is either not degraded, or only extremely slowly. Thorne (20) was able to isolate the enzyme system which catalyzes both the hydrolysis of poly-D-glutamic acid and also the formation of this polypeptide by transamidation. This enzyme system was found to hasten hydrolysis of synthetic poly (γ-D-glutamyl)-D-glutamic acid, poly (γ-L-glutamyl)-L-glutamic acid, and poly (α-L-glutamyl)-L-glutamic acid as well as the natural polypeptide, but would not cause hydrolysis of synthetic poly (α-D-glutamyl)-D-glutamic acid. These results

would again suggest the probable presence of gamma bonds in the polypeptide.

Bovarnick, et al. (21) have calculated the molecular dimensions of natural poly-D-glutamic acid of molecular weight 12,000 from sedimentation and diffusion measurements in the ultracentrifuge. The reported dimensions were 11 x 150-250 Å. No attempt was made to translate these dimensions into a configuration involving one or the other type of bond.

Ivanovics and St. Horvath (22) reported that the polypeptide does not exist as the free acid, but that the carboxyl groups not used in the peptide bonds are involved in hydrogen bonding. Such interactions of the carboxyl hydrogens would be expected to add stability to the alpha helical structure. Doty and co-workers (23) reported this year that poly-L-glutamic acid assumed the alpha helical configuration in solution until about forty per cent of the free carboxyl groups were ionized. The electrostatic force due to the carboxyl anions present at forty per cent ionization was calculated to be equal to 300 calories per residue. At this point the helix rapidly broke up to form random coils. The shift in structure occurred almost completely within the pH range 5.4 to 6.4. This is a reversible shift. Apparently the undissociated carboxyl hydrogens contribute considerable stability to the helical structure through hydrogen bonds. The results of infra-red studies, intrinsic

viscosity, light scattering, and change in optical activity are used to support their picture of the transition between helical and random coil structures. The pH dependence of specific optical rotation in saline-dioxane solvent mixture is also presented.

At the time of selection of this problem, little published information on the size and shape of the molecules, optical activity, or potential uses of the polypeptide was available. These problems, along with the problem of linkage, were studied. The methods of attack and the results obtained are described and discussed in the following chapters.

CHAPTER II

EXPERIMENTAL

Source of bacterial cultures. Through the courtesy of Dr. W. C. Haynes of the Northern Utilization Research Branch, a fresh slant of Bacillus licheniformis NRRL B-571 (formerly called B. subtilis Ford strain NRRL B-571) was obtained from the United States Department of Agriculture's stock culture collection in Peoria, Illinois. Fresh culture was also obtained by inoculating tubes of sterile broth with acetone dried pellicle from previous fermentations. In November, 1956, a glass vial of vacuum packed lyophilized spores and cells of B. licheniformis NRRL B-571 was received from the stock culture collection in Peoria. The lyophylized material from this vial was used to inoculate three tubes of sterile Sauton's medium. The growth resulting from the two preparations containing spores seemed luxuriant; each inoculum produced thick mats or pellicles of bacteria on the surfaces of the media. The mats had the typical pink to lavender coloration which is usually associated with peptide production. The growth resulting from inoculation with the bacteria from the fresh slant was

not so luxuriant and did not exhibit the colored pellicle. When the three sources of inoculum were checked for polypeptide production, the two regenerated from spores showed a good production, while that from the agar slant produced no poly-D-glutamic acid. Accordingly, this source of inoculum was not used further. The other cultures, those started from spores, were kept on slants covered with sterile mineral oil.

Determination of yield. The medium used for the production of poly-D-glutamic acid was a modified Sauton's medium (24), in which the 4 g. of L (+) glutamic acid was replaced by 4 g. of D,L-aspartic acid. Good growth on this medium is evidenced by a heavy pink pellicle floating on the surface of the liquid. Twenty liters of the medium was distributed in equal quantities in ten diphtheria toxin bottles of five liter capacity. The bottles were then plugged with cotton and autoclaved at 16 psi. for thirty minutes. The medium was allowed to stand for several days to ascertain its sterility. Each bottle was inoculated with about 2 ml. of the actively growing culture, followed by seven days of incubation at 37-8° C. The bottles were then taken from the incubator and the contents filtered through nylon parachute cloth into a large battery jar. The filtrate was stirred with Johns-Manville Celite 521 filter aid and filtered through a Buchner funnel to obtain a clear filtrate. The filtrate had a pH of about 6. Ionic material

was then removed from the filtrate by passing it through a cationic exchange column and then through an anionic resin column. The resins used were Amberlite IR-120, in the acid form, and IRA-410, in the hydroxyl form.* The polypeptide emerged from the cationic resin in opalescent solution at a controlled rate of flow with a pH value of 3. The effluent from the first column was immediately passed through the anionic resin, where it became more opalescent, sometimes to the point of incipient precipitation, but did not change appreciably in acidity. The solutions were washed through each column with distilled water.

The effluent from the anion exchange column still contained nonionic impurities. The polypeptide was removed from these by precipitation as the copper (II) salt. The solution from the exchange columns was stirred with sufficient ammonium hydroxide to bring the pH to about 6. Copper (II) sulfate pentahydrate was added as a saturated aqueous solution in an amount of 50 ml. per liter of treated medium with vigorous stirring. The precipitate of copper poly-D-glutamate began to form immediately but the stirring was continued for several hours to facilitate coagulation. The mixture was then allowed to sit undisturbed overnight before separating the solid from the supernatant. The solid was stirred with several portions of acetone on a Buchner funnel

*The Amberlite resins are products of the Rohm and Haas Co., Philadelphia, Pennsylvania.

and sucked dry. The product at this point was 36 g. of light green powder.

The copper peptide was next dissolved in a water solution of versene (ethylenediamine tetraacetic acid, disodium salt) having a weight of dissolved versene equal to twice the weight of the copper peptide added. The deep blue solution was filtered through paper and the residue re-extracted with aqueous versene until no more blue color remained. The combined extracts were then passed first over the anion exchange resin followed by passage through the cation resin. Passage over the cationic resin first seemed to cause precipitation of the versene in the column. This action was avoided by passing the solution over the anionic resin first, followed by passage over the cationic resin. The final effluent was clear and colorless and exhibited a pH of about 3.

The solution of poly-D-glutamic acid was concentrated by pervaporation. Three-foot lengths of viscose casing of about 3 inch diameter were filled with the polypeptide solution and hung in an air stream. The water diffused through the pores and evaporated from the outside of the casing. The evaporation cooled the casing and contents to about 10° C. The polypeptide was unable to pass through the pores of the casing and was thus concentrated in the interior of the tube. After the total volume had been reduced by about 9/10, the concentrated solution was dis-

tributed in 100 ml. portions to 500 ml. round bottom flasks and was shell-frozen. A mixture of dry ice and acetone was used as the freezing solution. The flasks were then connected to a VirTis Freeze-Dryer which was further connected to a high vacuum pump through a cold trap and a moisture trap. A vacuum of 10-20μ, as measured by a McLeod gage, was maintained until the contents of the flasks were completely dry. The yield of fluffy, white poly-D-glutamic acid recovered from the freeze-drying apparatus was 1.125 g. per liter of medium.

Soil conditioning tests. Most of the tests used to determine soil porosity and soil stability require specialized equipment which is not available in this department. Accordingly, arrangements were made through Dr. C. R. Russell, of the Forage and Agriculture Residues Section of the Department of Agriculture, to test our samples of the polypeptide and its salts for their effects on soil stability. A sample of nine grams of poly-D-glutamic acid was mixed as well as possible and divided into three equal portions. One portion was packaged and labeled poly-D-glutamic acid. A second portion was dissolved in water, with difficulty because part of the material had been dried to a glassy state, and titrated with sodium hydroxide solution to pH 7.0 $\pm$ 0.2. The third portion of the polypeptide was also dissolved in water and neutralized with a slurry of calcium hydroxide. A Beckman model G pH

meter was used to determine the end-points. Each of these two clear, colorless solutions was then lyophilized and the dry product packaged. They were designated the sodium and calcium salts of poly-D-glutamic acid, respectively. These three samples were then evaluated in soil tests. Miami silt loam, used as the test soil, was prepared according to the directions of Hedrick and Mowry (25). The soil crumbs of uniform size used in the wet sieve analysis were prepared as described by Geoghegan and Brian (26). The wet sieve analysis was carried out in duplicate on forty-gram portions of crumbs following the method of Yoder (27) using sieves with 0.84, 0.42 and 0.25 mm. openings. The test results are summarized in Table 2.

TABLE 2

RESULTS OF SOIL STABILITY TEST

Soil Conditioner	Percentage water stable aggregates 0.25 mm. Material applied as: Powder 0.1g/100g.	Aqueous sol'n 0.25g/100g.
Sodium Polyglutamate	69.5	87.3
Calcium Polyglutamate	53.4	83.0
Polyglutamic Acid	50.5	83.0
Krilium (CRD 186)	85.3	100.0
Control	1.0	---

Synthesis of D,L-2-phthalimidoglutaric anhydride.

The method of King and Kidd (28) was first used for the synthesis of this compound. The desired product was obtained in 64% yield. (King and Kidd report 74% yield.) The yield was improved by making some changes in the procedure. Ten grams (0.068 mole) phthalic anhydride and 10 g. (0.068 mole) L-glutamic acid were added to 40 ml. pyridine and the mixture maintained at reflux for two hours. Only a small amount of solid had not dissolved by the end of this time. The pyridine was then distilled off under partial vacuum and the remaining syrup boiled with acetic anhydride (30 cc.) for about five minutes. The remaining acetic anhydride and acetic acid were then distilled off under reduced pressure. During this distillation, a solid separated from solution. The residue was cooled and ether was added in excess. The suspension of solid in ether was kept in the refrigerator overnight. The solid was then filtered off and washed three times with dry ether. The white solid was then air dried for a day. There remained 13.2 g. (75% yield) of D,L-2-phthalimidoglutaric anhydride having a melting point of 198-201° C. King and Kidd reported a melting point of 195-6° C. and Sheehan and Bolhofer reported 204-5° C. The method of synthesis reported by Sheehan and Bolhofer (29) was also tried and found to be ineffective in our hands; no yield of the desired product was obtained.

Synthesis of γ-methyl ester of 2-phthalimidoglutaric acid. To 20 ml. dry methanol was added 5 g. (0.018 mole) of the anhydride prepared above. The solution was refluxed two hours and then distilled under reduced pressure until all the methanol was removed. The remaining syrup was dissolved in saturated aqueous sodium bicarbonate. Concentrated hydrochloric acid was added to the solution to a congo red end-point. Sometimes the product precipitated at this point, but usually it was extracted from the water solution with ether. The ether extracts were combined and dried over anhydrous sodium sulfate. The dry ether was allowed to evaporate and the residue recrystallized from ether. A white solid having a melting point of 113-4° initially and 115-7° C. after two more recrystallizations from ether was obtained in 70% yield. Neither of the methods reported by Sheehan and Bolhofer, and King, et al. (30) gave the reported yields when followed closely. King, et al. reported a melting point of 114°, while Sheehan and Bolhofer reported 119.5-120.5° C.

Attempted synthesis of α-methyl ester of 2-phthalimidoglutaric acid. The product obtained by the method of Sheehan and Bolhofer for the synthesis of α-methyl 2-phthalimidoglutarate was white and had a melting point of 105-120° C. (Cf. reported m.p. 154-5° (29)), which could not be raised by recrystallization. The same product was obtained using either freshly prepared sodium methoxide or

commercial sodium methoxide as the cleaving agent for the anhydride.

Attempted reduction of the free carboxyl group of γ-methyl-2-phthalimidoglutarate. Three grams (0.01 mole) of the γ-methyl-2-phthalimidoglutarate was dissolved in 25 ml. anhydrous dioxane (31) with warming. To the warm solution was added 2.4 g. (0.02 mole) thionyl chloride and the warming continued for one half hour. At the end of this time the open flask was heated to a temperature just short of the boiling point of dioxane in order to distill out any excess thionyl chloride along with gaseous products. Heating was continued for two hours. The flask and contents were then cooled to 0° C., and 0.38 g. (0.01 mole) powdered sodium borohydride was added. The addition was made cautiously and gradually. Gradual warming to room temperature did not give rise to vigorous reaction. The suspension was then heated on a steam bath for 30 minutes, cooled, and water was cautiously added with vigorous stirring. Hydrogen was evolved. The cooled solution was acidified and then extracted three times with ether. The combined ether extracts were dried over sodium sulfate and then allowed to evaporate to dryness. The residue was dissolved in the minimum amount of acetone and allowed to crystallize in the freezer. Collection of the crystals on a Hirsch funnel, followed by a brief washing with cold ether, yielded 0.74 g. of white powder melting at 125° C. after drying at room

temperature for a day. This represented a 27% yield of D, L-methyl-4-phthalimido-5-hydroxyvalerate.

Removal of the phthaloyl group of methyl-4-phthalimido-5-hydroxyglutarate. Two methods (28, 30) were modified to effect the removal of the phthaloyl protecting group from the nitrogen. Thirty milliliters of ethyl alcohol was used as the solvent for 2.8 g. (0.01 mole) of the reduced product described above and 14 ml. of 1 M. hydrazine hydrate in ethanol. This mixture was allowed to stay in a stoppered flask with occasional shaking for two days at room temperature. The solution was then made acid to congo red with concentrated hydrochloric acid. The precipitate of phthalhydrazide was filtered off and the filtrate was evaporated to dryness. Only a very small amount of solid remained. The residue was washed on a filter with cold ether. After drying at room temperature for several days, the remaining white crystals melted at 190-5° C. About one milligram of crystals was obtained. This product was believed to be D,L-methyl-4-amino-5-hydroxyvalerate.

Determination of optical rotation. The optical rotation measurements were made with a Gaertner L-320 polarimeter. A sodium vapor lamp was the source of illumination. A two decimeter, 3 mm. bore tube of glass was used in all of the determinations. A water-jacketed tube of these dimensions, necessary to conserve the limited stock of poly-D-glutamic acid, was unavailable. The practice was there-

fore to keep the tube stored at an ambient temperature not far from that of the constant temperature bath (20° C.). The solutions of polypeptide adjusted to proper pH, were immersed in the constant temperature bath for approximately 15 minutes before filling the polarimeter tube. The readings of rotation were taken very shortly after filling the tube in an effort to keep the change in temperature of the polypeptide to a minimum. No change in rotation during a series of readings on any sample, which might have been due to temperature change, was noted. A polarizing prism angle of 2° was found to give the narrowest range of extinction consistent with readability, and was used throughout the study. The solutions for measurement of optical rotation were prepared by diluting a 1 ml. aliquot of a stock solution of 0.2 g. poly-D-glutamic acid in 10 ml. distilled water with various proportions of 0.4N sodium hydroxide solution and distilled water to a total volume of 5 ml. The pH values of the solutions in the first series were read with a battery operated pocket pH meter manufactured by Analytical Measurements, Inc. The specially constructed semi-micro size probe unit of this pH meter failed after some use and a replacement could not be obtained. No other operable pH meter was available with facilities for measuring the pH values of samples of about 3 ml. The second series of determinations was therefore made using short range pHydrion papers. The accuracy of these papers was about ± 0.2 pH unit. The two

series of readings were made at concentrations of poly-D-glutamic acid in the range of 4-5 mg./ml. A third series was made at a concentration of 16 mg./ml. See Table 3 and Figure 1.

TABLE 3

OPTICAL ROTATION OF POLY-D-GLUTAMIC ACID

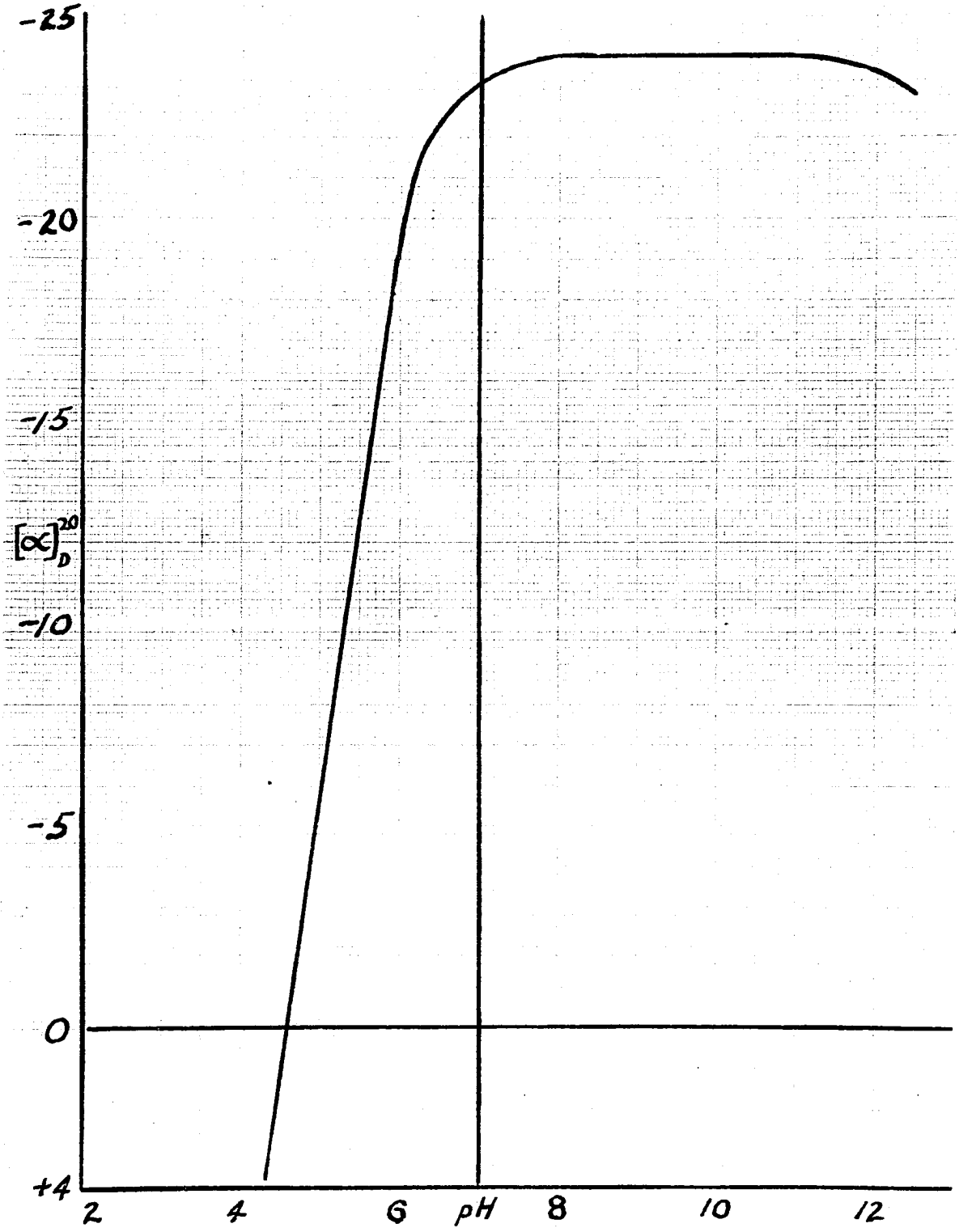
pH	Peptide Concentration (mg./ml.)	Observed Rotation (Degrees)	(Degrees)	pH Measurment *
1	4.060	+0.158	+19.46	m
1	"	+0.155	+19.09	m
3.65	20.300	+0.577	+14.21	m
4.6	5.232	-0.073	- 7.00	p
4.75	4.840	-0.013	- 1.29	p
4.8	16.014	-0.423	-13.19	p
4.85	4.060	-0.042	- 5.17	m
5.3	4.840	-0.174	-18.00	p
5.8	5.232	-0.137	-13.05	p
5.85	4.018	-0.182	-22.65	m
6.0	5.232	-0.155	-14.81	p
6.00	4.060	-0.146	-17.98	m
6.0	16.014	-0.585	-18.27	p
6.4	5.232	-0.203	-19.43	p
6.5	4.840	-0.183	-18.95	p
6.8	5.028	-0.217	-21.55	p
6.8	5.232	-0.240	-22.94	p
7.2	5.028	-0.227	-22.54	p
7.6	5.232	-0.247	-23.57	p
8.2	4.840	-0.190	-19.63	p
8.5	"	-0.191	-19.70	p
8.5	5.028	-0.237	-23.54	p

TABLE 3 - Continued.

pH	Peptide Concentration (mg./ml.)	Observed Rotation (Degrees)	(Degrees)	pH Measurment *
8.7	5.028	-0.250	-24.86	p
8.8	16.014	-0.682	-21.28	p
8.8	"	-0.682	-21.28	p
9.1	4.840	-0.211	-21.65	p
9.10	4.018	-0.187	-23.27	m
9.3	5.028	-0.239	-23.79	p
9.4	16.014	-0.673	-21.02	p
10.3	5.232	-0.243	-23.25	p
10.4	5.028	-0.273	-27.19	p
10.5	16.014	-0.674	-21.04	p
10.8	4.840	-0.140	-18.11	p
10.8	"	-0.187	-19.29	p
10.8	16.014	-0.697	-21.70	p
10.8	5.028	-0.238	-23.70	p
11.10	4.018	-0.191	-23.77	m
11.25	4.060	-0.163	-20.07	m
11.3	16.014	-0.700	-21.68	p
11.40	4.060	-0.198	-24.38	m
11.60	"	-0.197	-24.26	m
13.0	4.840	-0.201	-20.70	p
13.0	5.028	-0.208	-20.71	p
13.2	"	-0.193	-19.22	p
13.3	16.014	-0.573	-17.90	p

*The pH values of the solutions were measured by either pHydrion paper (p) or Beckman Model G pH meter (m).

Figure 1



VARIATION OF SPECIFIC ROTATION WITH ACIDITY

Electron microscopy. The console model RCA electron microscope of the University of Oklahoma Research Institute was used for this study. The instrument had been in semi-continuous operation previous to this use. All necessary adjustments and instructions were provided by Mr. Robert Houston of the Research Institute staff.

The polypeptide and its salts were prepared for viewing in the electron microscope by dissolving them in water. One drop of the appropriate solution was placed on the screen and the water allowed to evaporate in a desiccator at room temperature. It was found towards the end of the study that larger fibers were formed by allowing the water to evaporate in an 80° C. oven. In either case, the drying did not take long. No attempt was made to keep the various solutions of poly-D-glutamic acid and its salts at a uniform concentration. However, dilute solutions were used in all cases.

The screens were prepared by the following procedure. Circles of 200 mesh screening were purchased for this use. Formvar film approaching monomolecular thickness, and prepared by spreading a drop of formvar dissolved in an organic solvent on the clean surface of water, was then placed on the mesh circles. This combination of formvar film and wire screen was dried before use.

Electron Photomicrographs. The dried screens holding the polypeptide were inserted in the electron path

in the microscope and a high vacuum obtained in the system. Crystallinity was apparent in the resultant electron photomicrographs by virtue of the fibrous structure of the polypeptide. Typical fibers are shown in Figures 2, 3, and 4. No attempt was made to measure the length of the fibers, which varied from less than one screen diameter to more than four diameters. Average widths of the fibers were measured by projecting the photomicrographs with a slide projector and measuring the distances with a cathetometer. The magnification factor was obtained by measuring photomicrographs of polystyrene latex particles. The latex particles were supplied by The Dow Chemical Company and were certified to have a diameter of 5570 Å $\pm$ 108 Å. The average widths of the fibers measured were 900 Å, 1050 Å, and 680 Å for the sodium salt, calcium salt, and free acid form of poly-D-glutamic acid, respectively. The somewhat regular bands appearing in the fibers of one photomicrograph of the sodium salt had an average width of 125 Å.

Electron diffraction patterns. Attempts were made to obtain diffraction patterns for the peptide and its sodium, calcium, and copper salts. Only one clear multiring diffraction pattern was obtained, and this was from an apparently amorphous pile of calcium polyglutamate. All other samples gave diffuse single-ring diffraction patterns, which appeared to be the same as the diffraction pattern of the formvar base.

Figure 2

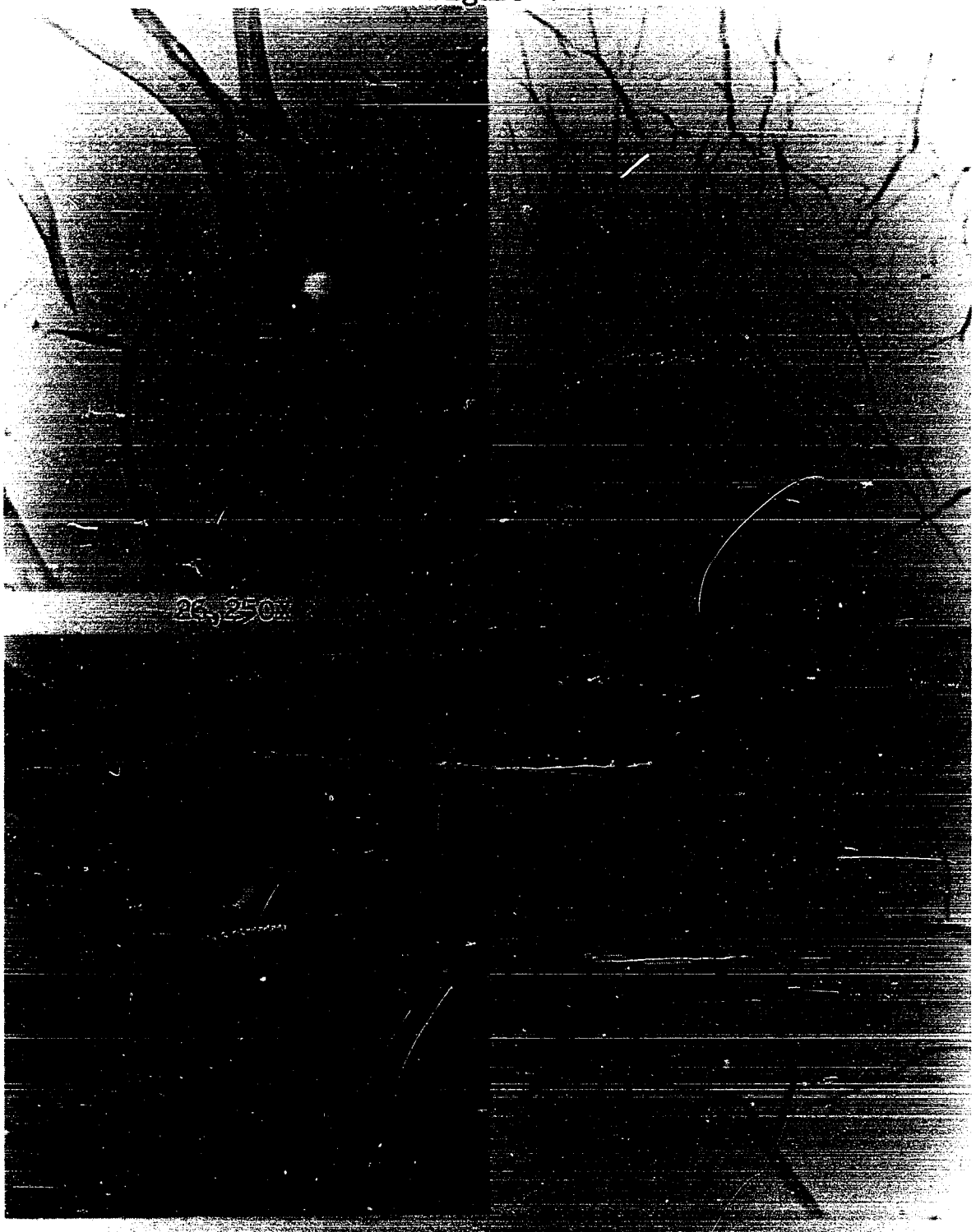
ELECTRON PHOTOMICROGRAPHS OF
SODIUM POLY-D-GLUTAMATE FIBERS

Figure 3

26,250X 2625X

ELECTRON PHOTOMICROGRAPHS OF CALCIUM POLY-D-GLUTAMATE FIBERS

Figure 4



26,250X 26,250X

ELECTRON PHOTOMICROGRAPHS OF POLY-D-GLUTAMIC ACID

Ultracentrifuge studies. The behavior of twenty-seven solutions of polyglutamic acid were studied with the Spinco Model E ultracentrifuge. In all cases the concentration of the polypeptide or its salt was 1%. The varied conditions applicable to the samples are summarized in Table 4.

The prepared solutions were clarified by filtration or ultracentrifugation at about 20,000 rpm. before being analyzed at the higher speeds. Samples 1 through 22 were spun at 59,780 rpm. and samples 23 through 27 at 52,640 rpm.

The densities of samples 7 through 22 were determined using a standard pycnometer and their viscosities calculated from the lengths of time required to flow through a standard viscometer. The viscosities, densities, and ultracentrifuge data were substituted into the equation (32) for the sedimentation constant. Values for the apparent diffusion coefficients were calculated from measurements of the sedimentation curves according to the instructions of Schachman (33). The sedimentation constants and apparent diffusion coefficients were then combined with temperature and density data to provide apparent molecular weights (34). These calculated molecular weights ranged from 240,000 for sample number 19 to 460,000 for sample number 14. A molecular weight of 32,000 for sample number 14 was also calculated using, instead of an apparent diffusion coefficient, a value of 3 for f/f_o, the frictional force ratio. This

- TABLE 4

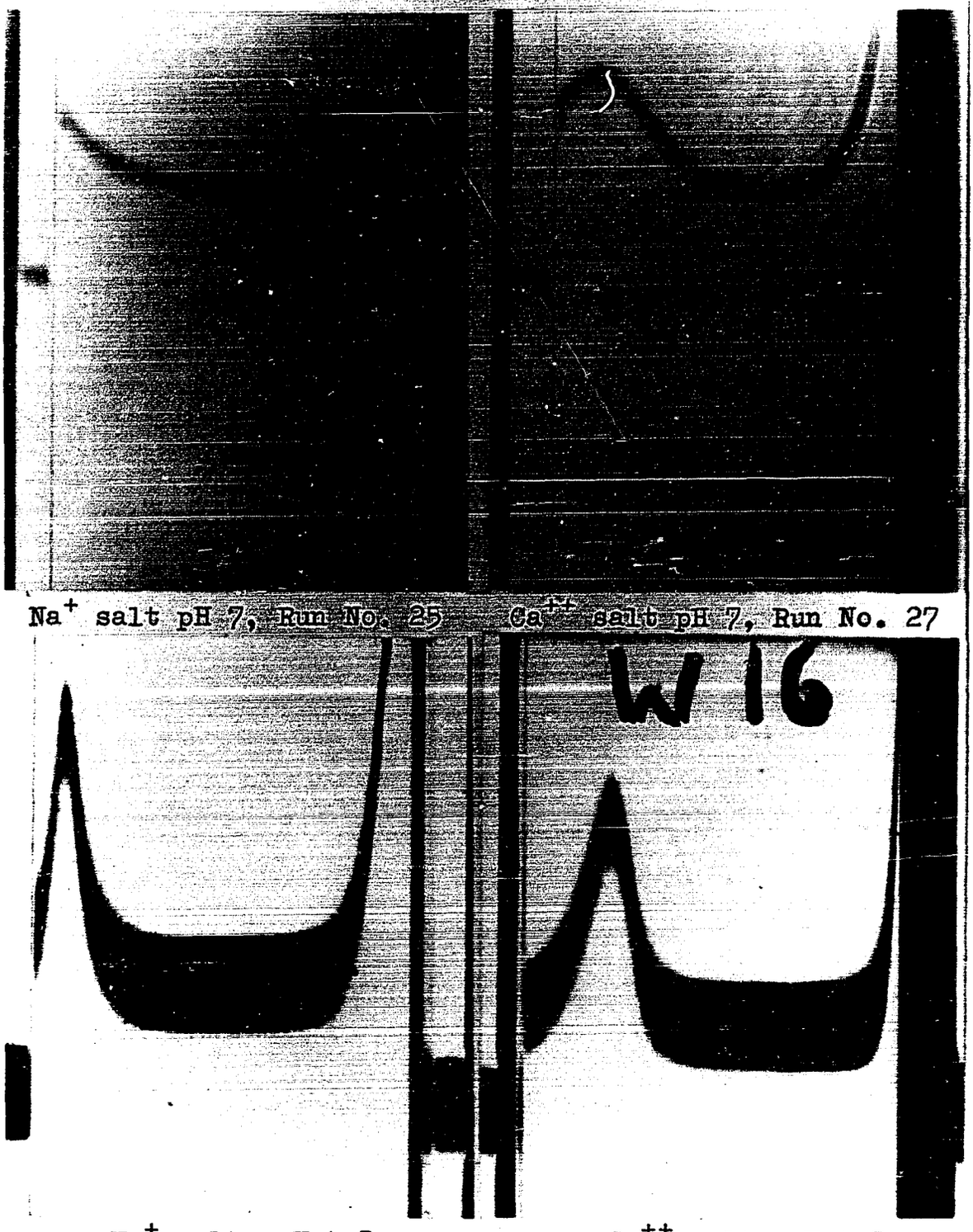
SUMMARY OF THE ULTRACENTRIFUGE RUNS

Run No.	Peptide Form	Acetate Buffer (0.1N)	pH	NaCl Conc. (Molarity)	Sedimentation Coefficient $S_{20,w} \times 10^{13}$
1	H	Yes	2.5	0	
2	H/Na	Yes	3.5	0	
3	H/Na	Yes	4.5	0	
4	Na	Yes	7.0	0	
5	H	Yes	2.5	0	
6	Ca	Yes	4.5	0	
7	H	No	1.	0	
8	H	No	2.	0	
9	H	No	2.5	0	
10	H	No	1.	0.05	
11	H	No	1.5	0.05	
12	H	No	1.	0.1	
13	H	No	1.5	0.01	
14	H	No	2.8	0.025	1.58
15	H	No	2.7	0.05	1.67
16	H	No	2.7	0.1	1.87
17	H	No	2.6	0.3	1.90
18	H	No		0.525	1.89
19	H	No	2.5	1.	1.92
20	H	No	2.4	1.5	1.97
21	H	No	2.4	2.	2.11
22	H	No	2.3	2.5	1.29
23	H	No	3.6	0	
24	Na	Yes	4.6	0	
25	Na	No	7.	0	
26	Ca	Yes	4.6	0	
27	Ca	No	7.	0	

value of f/f_o was obtained from a graph of the variation of f/f_o with changes in dimensions of the molecules as presented by Svedberg and Pedersen (35). The dimensions chosen, 11 x 250 Å, were reported by Bovarnick, et al. (21).

Selected ultracentrifuge curves are presented in Figures 5, 6, and 7.

Figure 5



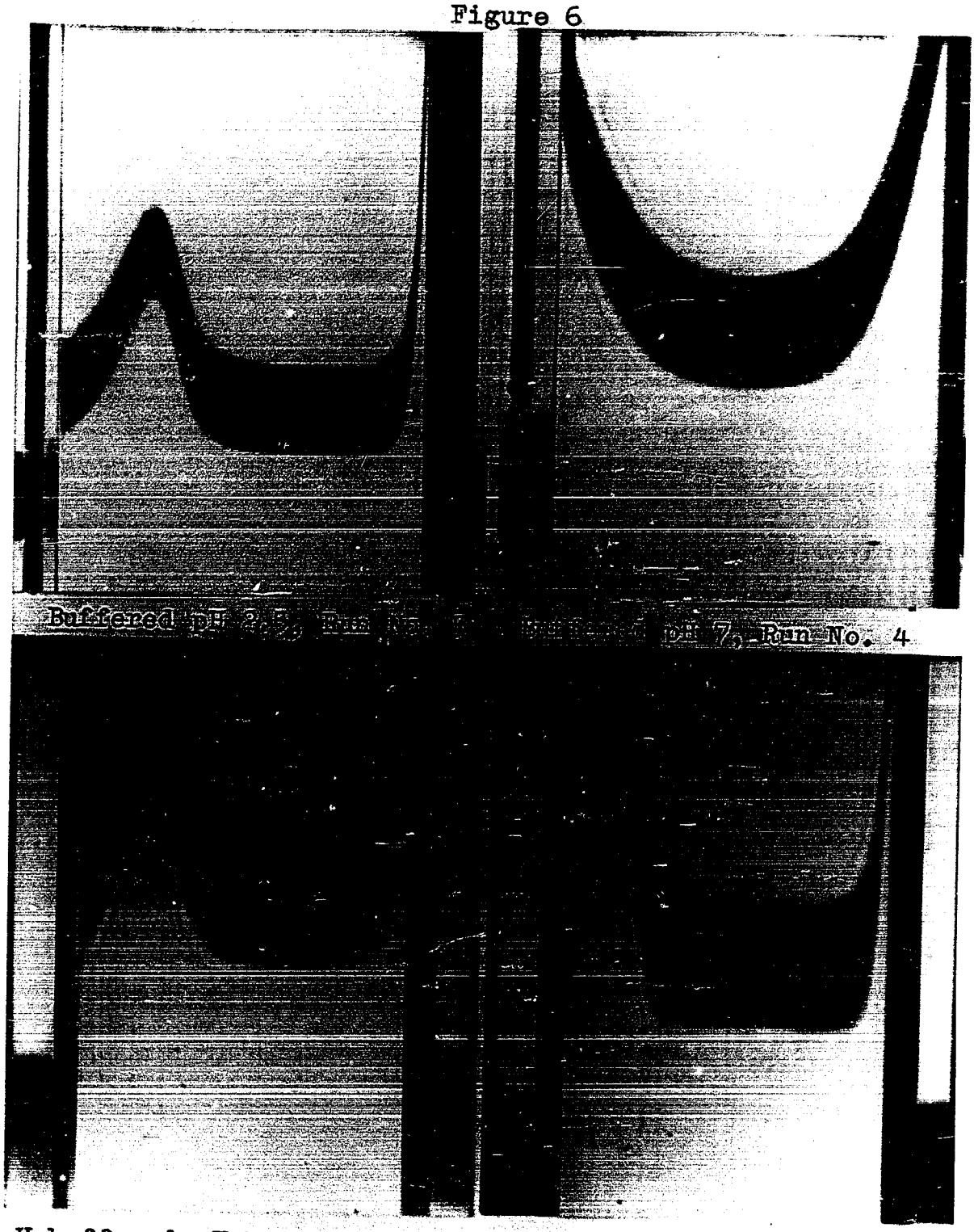
Na^+ salt pH 7, Run No. 25 Ca^{++} salt pH 7, Run No. 27

Na^+ salt, pH 4.5
Run No. 3

Ca^{++} salt, pH 4.5
Run No. 6

VARIATION OF SEDIMENTATION WITH CATION PRESENT

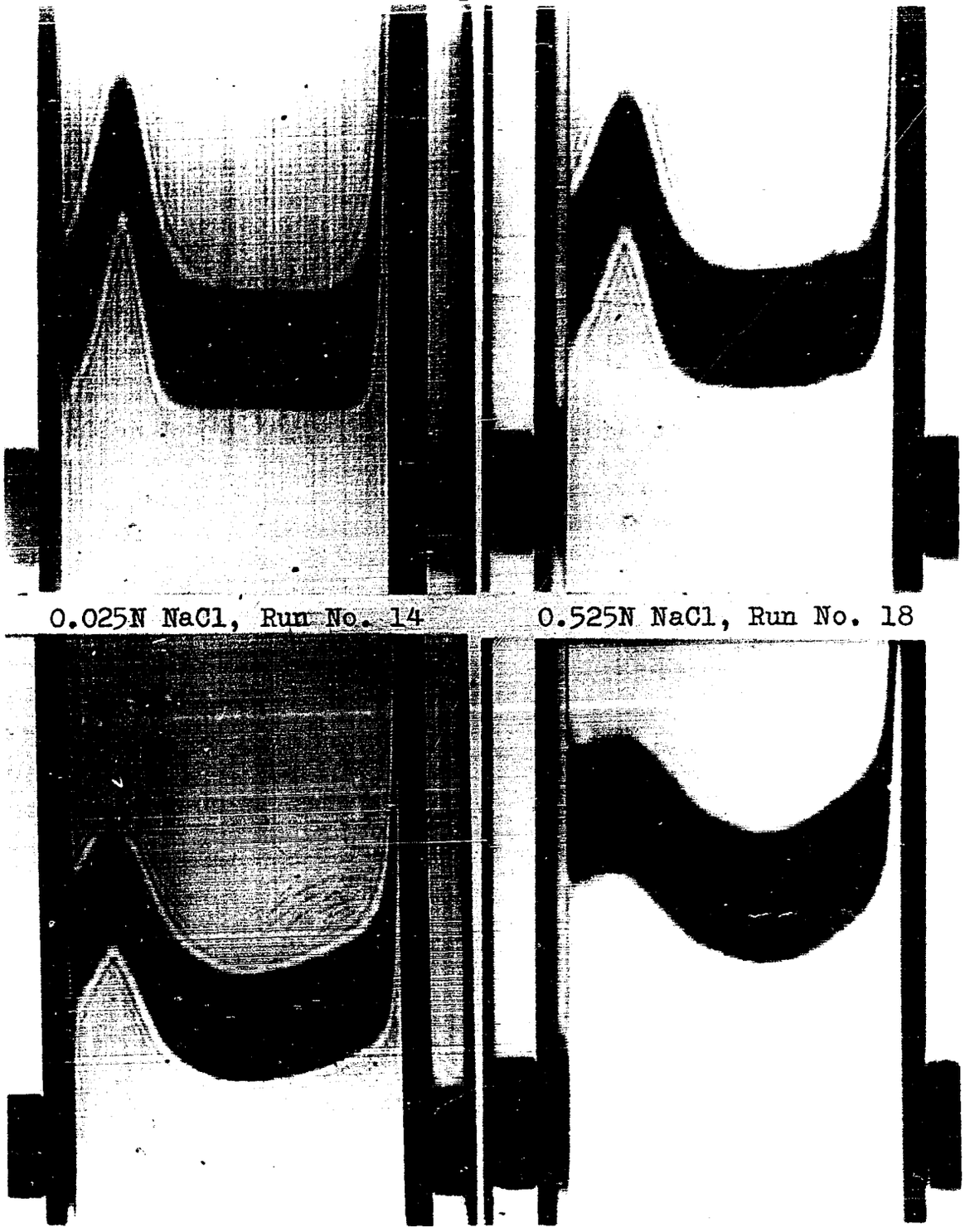
Figure 6



Unbuffered pH 1, Run No. 7 Unbuffered pH 2.5, Run No. 9

VARIATION OF SEDIMENTATION WITH ACIDITY

Figure 7



0.025N NaCl, Run No. 14 0.525N NaCl, Run No. 18

2N NaCl, Run No. 21 2.5N NaCl, Run No. 22

VARIATION OF SEDIMENTATION WITH SALT CONCENTRATION

CHAPTER III

DISCUSSION

It was found that as the bacterial culture of Bacillus licheniformis was continuously transferred on agar slants, it lost its ability to synthesize poly-D-glutamic acid on the modified Sauton's medium. Similar findings were reported subsequently by Thorne (20). The storage of the stock culture on agar slants covered with sterile mineral oil seems to cause the metabolic processes of the bacteria to slow down. The cultures do not have to be transferred as often under these conditions, and thus the rate of loss of synthetic ability is not as great. The regeneration of the cultures from spores seems to be the best solution.

The relative yield of polypeptide from the culture medium is of interest purely as comparison with other methods and also as an economic factor in the possible use of this material as a soil conditioning agent. Average yields of 2-3 g. polypeptide per liter of modified Sauton's medium supporting growth of B. subtilis in static culture were reported by Thorne, et al. (20, 36). They also reported yields as high as 15 g. per liter of a special medium supporting

growth in aerated submerged culture. There is some evidence that the product in this case contained only about 50% D-glutamic acid, the remainder being the L-isomer. Watson, et al. (37) reported the isolation of a polyglutamic acid from B. subtilis cultures which contained only 85% of the D-isomer.

The yield reported herein is the result of the first continuous preparation and isolation of poly-D-glutamic acid using the method of isolation involving versene and ion exchange resins. Other methods employ a step or steps involving contact of the polypeptide with acid for varying lengths of time, usually in the form of dialysis against acid after sulfide precipitation of the copper from the copper polyglutamate. Hanby and Rydon (7) showed that contact with mild acid leads to considerable hydrolysis. The method of isolation presented herein avoids prolonged exposure to strong acid and is considered to be much preferable to others.

Since the organisms capable of producing poly-D-glutamic acid are primarily soil bacteria, it was thought that perhaps the product is formed to stabilize the soil in some way; perhaps it leads to better aeration of the soil by forming aggregates of soil particles. This would be an action analogous to that of the soil conditioner known as Krilium, which is a pure synthetic product. Polyglutamic acid and its salts were found to be more than 80% as effec-

tive as Krilium in the ability to stabilize soil by aggregation of the small silt particles. Whether or not the bacteria produce this peptide for the purpose of improving the soil has not been answered, but the polypeptide does improve the soil!

The question of which carboxyl group is involved in the peptide bonds has been studied by various methods. The direct chemical approach would seem to be the best, however. Kovacs and co-workers (15, 16, 17) and Brown (18) both reported such attempts, but the results are inconclusive. Accordingly, considerable work was done in an attempt to set up proof of bonding by reducing the free carboxyl group and isolating and identifying the product(s). Thus, a polypeptide having the α-carboxyl groups free would be reduced and hydrolyzed to 4-amino-5-hydroxyvaleric acid. If the γ-carboxyl groups were free, 2-amino-5-hydroxyvaleric acid would result. These two compounds could each be estimated without separation from one another by using two different reagents; periodic acid would titrate the former but would not affect the latter, and ninhydrin would react with the latter without being hindered by the first compound.

It seemed necessary to first synthesize these two compounds as standards for comparison before attempting the reduction of the polyglutamic acid. The published reports of synthesis of Sheehan and Bolhofer (29), of King and Kidd (28), and of King, Jackson and Kidd (30) made the task appear

to be an easy one. Attempts at repeating all parts of the work of Sheehan and Bolhofer were completely unsuccessful. Two other methods had to be modified to give adequate results. The conditions of the reduction step were based upon general recommendations of the hydride manufacturers. In general, the reduction seemed easy and the yield high, but the product of the reduction was used up quickly in attempting the next synthesis step and no rigorous proof of the composition of the compound was obtained. The amount of the methyl ester of 4-amino-5-hydroxyvaleric acid obtained was barely sufficient for the determination of the melting point. This area of the problem is very interesting and requires further research.

It had been observed that precipitation of the copper salt of poly-D-glutamic acid at pH 3 gave a gummy product which could be stretched to form films and threads. Attempts were made to dry such stretched threads under tension and to see if orientation into fibers could be observed in the electron microscope. Simple drying of solutions was found to give the observable fibers. The fiber sizes are larger than the molecular dimensions reported for poly-D-glutamic acid by Bovarnick, et al. (21). The fibers do seem to be quite uniform. The internal periodic structure seen in two of the fiber preparations is interesting and suggestive of a complex composition in the fibers.

The diffraction patterns were also desirable as

possible sources of information concerning the molecular structure of poly-D-glutamic acid and its salts. It was soon established that diffraction patterns were difficult to obtain photographically. Attempts were made to eliminate the formvar supporting film, but these were not fruitful.

The optical rotation studies were carried out at low concentrations to avoid the greater opacity of solutions of higher concentrations. The short supply of poly-D-glutamic acid also dictated the low concentrations used. The series of readings taken at higher concentration of polypeptide show the accuracy of the readings of the first two series. It is to be noted that the curve of specific rotation versus pH values for poly-D-glutamic acid is very similar to the curve presented by Doty, et al. (23) for a poly-L-glutamic acid. The similarity includes the minimum in alkaline solution, the maximum in acidic solution and the flat portion of the curve after a certain ionization has been reached. The solvent system in this case was a one to two mixture of dioxane and 0.2 M. solution of sodium chloride in water. Sachs and Brand (38) have presented specific rotation values of some synthetic di- and tripeptides of certain amino acids taken in 0.5N hydrochloric acid solutions. The gamma linked dipeptide of L-glutamic acid showed a rotation of $+3.8^{\circ}$, presumably at low pH values. The gamma linked tripeptide of L-glutamic acid showed a rotation of -7.2°.

The specific rotation curve presented in this paper exhibits a flat portion corresponding to the rotations in alkaline solution. This is to be expected because the poly-D-glutamic acid lacks free amino groups. The added alkali is actually only titrating the free acid groups, since this polypeptide does not have a large quantity of unused amino groups as does a normal protein. The usual protein curve exhibits a large change in rotation both in acid and in alkali. The curve presented by Doty and co-workers also has a flat portion because poly-L-glutamic acid does not have more than one free amino group per polypeptide unit either.

The specific rotations of most proteins show a quite small change with change in pH close to neutrality (39). A corresponding dip in the curve for poly-D-glutamic acid was absent. This absence was also noted in the curve presented by Doty and others. The slight saddle to be found in the normal protein curves is probably also due to a balance of free acid and free amino groups. Since the two polyglutamic acids do not have any free amino groups, the slight dip is not to be expected.

The change in specific rotation as the polypeptide is neutralized is essentially complete within a short pH range. This fact can be used to support the conclusion that a change in molecular configuration from an α-helix to a random coil occurs when the electrostatic forces between

carboxylate groups of the polypeptide become great enough.

Solutions of sodium poly-D-glutamate show a high viscosity. This is not true for solutions of the calcium salt or for poly-D-glutamic acid solutions. It has been postulated that the un-ionized free acid sediments most quickly because of lack of interaction between molecules. Calcium poly-D-glutamate also sediments rapidly because the calcium ion may cause association of the polyglutamate ions, thus increasing the effective molecular weight. Also since calcium is divalent, it would be expected to be more closely bound to the polyglutamate and therefore reduce electrostatic interaction between molecules. Sodium poly-D-glutamate would be expected to sediment more slowly because it is more highly charged and is subject to two braking effects: the anions interact electrostatically and become more extended, and their movement is hindered by the oppositely charged gegenions. Both of these interactions would tend to decrease the sedimentation value. These interactions are probably also the cause of the highly viscous nature of the solutions. Figure 5 shows the higher sedimentation velocity of the calcium salt over the sodium salt of poly-D-glutamic acid.

A similar effect would be expected to occur as the acidity changes. In acid solution, there is little ionization of the poly-D-glutamic acid and the sedimentation would be most rapid. At higher pH values, the polyglutamic acid would be more ionized and electrostatic repulsions would

greatly decrease the sedimentation velocity due to the extension of the polyglutamate ion. Such effects of acidity are graphically illustrated in Figure 6.

Sedimentation velocity measurements are frequently made on solutions of the substance in the presence of dissolved salts. It is felt that the salt eliminates or minimizes anomalous effects. It is seen from Table 4 that increased salt concentrations give rise to decreased pH values. The effect of varying salt concentration on sedimentation velocities is seen in Figure 7. A high salt concentration greatly decreases the liklihood of molecular interaction, since the relatively few polyglutamate ions are surrounded and effectively masked from one another by the large number of salt ions present. As the large polyglutamate anions fall through the solution, they are surrounded by fields of high concentrations of cations. The result is that the sedimentation is hindered. This appears as a decrease in the sedimentation velocity coefficient and a decrease in the efficiency of separation, i.e., the originally sharp boundary of separation rather quickly changes into a diffuse area. This can also be seen in Figure 7.

CHAPTER IV

SUMMARY

Bruckner, Ivanovics and Erdos (8, 9, 10) in 1937 first reported the isolation of a polypeptide of bacterial origin consisting only of levorotatory glutamic acid. It has been found that Bacillus anthracis, B. licheniformis, B. mesentericus and B. subtilis all produce poly-D-glutamic acid when grown on simple synthetic media. Isolation of the polypeptide from the culture medium yields a product of varying molecular size depending upon the conditions of growth and isolation.

The polypeptide is particularly interesting for polyelectrolyte studies because of its simple constitution. It has been proposed as a starting material for blood plasma volume expanders. The dibasic character of glutamic acid raises the question of type of linkage in poly-D-glutamic acid. The linkage could involve either of the two carboxyl groups found on each glutamic acid unit.

The purpose of this research was to characterize poly-D-glutamic acid by optical activity, sedimentation and electron microscopy studies; to determine the efficiency of

the method of preparation; to determine the effectiveness of the polypeptide as a soil conditioning agent; and to study the problem of linkage by chemical means.

From the specific rotation values of poly-D-glutamic acid at various levels of acidity, it appears probable that the polypeptide normally exists at low pH values as a compact un-ionized unit, perhaps in the -helical configuration, and extends more or less randomly with increasing pH and ionization. The extension is probably due to electrostatic repulsions.

Results of sedimentation velocity studies with the ultracentrifuge support the theory of repulsion and extension just mentioned. Increasing alkalinity of the samples, as well as increasing salt concentration and replacement of the acid hydrogens of poly-D-glutamic acid by sodium ions, produce a decrease in the rate of sedimentation. Apparent diffusion coefficients ranging from 2.2×10^{-8} to 8.7×10^{-8}, sedimentation velocity constants ranging from 1.4 S to 2.1 S, and molecular weights ranging from 240,000 to 460,000 were calculated from the ultracentrifugation data.

Solutions of poly-D-glutamic acid and of its salts were found to yield uniform fibers upon evaporation of solvent. Measurement of the widths of these fibers with the electron microscope gave values of 900 Å for the sodium salt and very similar values for the other forms. Attempts were also made to study the electron diffraction of the

polypeptide.

The reported method of isolation of poly-D-glutamic acid using versene and ion exchange resins is thought to be an improvement over other methods reported in the literature. Yields as high as 1.125 g. poly-D-glutamic acid per liter of medium were obtained.

The sodium salt of poly-D-glutamic acid, the calcium salt and the free acid were found to be more than 80% as effective as Krilium CRD 186, a standard soil conditioning agent, in their action on aggregation of soil particles. This was found to be the case regardless of the manner of application of the agents.

The compounds, 2-phthalimido-D,L-glutaric anhydride, γ-methyl-2-phthalimido-D,L-glutarate, methyl-4-phthalimido-5-hydroxyvalerate, and methyl-4-amino-5-hydroxyvalerate, were prepared as intermediates for use in the study of the exact peptide linkage in poly-D-glutamic acid.

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